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Constitutive activation of T cells by γ2-herpesviral GPCR through the interaction with cellular CXCR4

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ABSTRACT

Members of the herpesviral family use multiple strategies to hijack infected host cells and exploit cellular signaling for their pathogenesis and latent infection. Among the most intriguing weapons in the arsenal of pathogenic herpesviruses are the constitutively active virally-encoded G protein-coupled receptors (vGPCRs). Even though vGPCRs contribute to viral pathogenesis such as immune evasion and proliferative disorders, the molecular details of how vGPCRs continuously activate cellular signaling are largely unknown. Here, we report that the vGPCR of Herpesvirus *saimiri* (HVS), an oncogenic γ2-herpesvirus, constitutively activates T cells via a heteromeric interaction with cellular CXCR4. Constitutive T cell activation also occurs with expression of the vGPCR of Kaposi's sarcoma-associated herpesvirus (KSHV), but not the vGPCR of Epstein-Barr virus. Expression of HVS vGPCR down-regulated the surface expression of CXCR4 but did not induce the degradation of the chemokine receptor, suggesting that vGPCR/CXCR4 signaling continues in cytosolic compartments. The physical association of vGPCR with CXCR4 was demonstrated by proximity ligation assay as well as immunoprecipitation. Interestingly, the constitutive activation of T cells by HVS vGPCR is independent of proximal T cell receptor (TCR) signaling molecules, such as TCRβ, Lck, and ZAP70, whereas CXCR4 silencing by shRNA abolished T cell activation by vGPCRs of HVS and KSHV. Furthermore, previously identified inactive vGPCR mutants failed to interact with CXCR4. These findings on the positive cooperativity of vGPCR with cellular CXCR4 in T cell activation extend our current understanding of the molecular mechanisms of vGPCR function and highlight the importance of heteromerization for GPCR activity.

1. Introduction

Herpesviruses are widespread mammalian pathogens that establish lifelong latent infections, possibly via a long evolutionary history of specific interactions with their host. The members of the herpesviral family use multiple strategies to hijack infected host cells and exploit cellular signaling to ensure successful infection and propagation. One of these strategies includes encoding multiple regulatory viral proteins, such as cytokines, chemokines, and viral G protein-coupled receptors (vGPCRs) in their genomes. All the viral genomes of β- and γ-herpesviruses encode at least one vGPCR which is homologous to cellular chemokine receptor [1,2]. vGPCRs were probably derived from the host genome during evolution and modified to support various aspects of the viral lifecycle, concomitant with viral pathogenesis such as immune evasion and proliferative disorders. In contrast to host chemokine receptors, which are activated in a ligand dependent manner and predominantly couple to $G\alpha_{i/o}$ proteins, several vGPCRs are constitutively active and can couple to multiple G proteins, thereby harnessing GPCR signaling in host cells and organisms [1,2]. Since some naturally occurring, constitutively active cellular GPCR variants have been shown to be associated with diverse pathophysiological conditions in humans [3], the constitutively active vGPCRs might play a crucial role in viral pathogenesis and could be drug targets [2]. Indeed, constitutively active vGPCR of Kaposi's sarcoma-associated herpesvirus (KSHV), a member of γ-Herpesvirus, is causally associated with the initiation and progression of Kaposi's sarcoma [4,5]. KSHV vGPCR is capable of activating various signaling pathways, including the PI3K/AKT/mTOR axis for cellular proliferation [6,7], and leads to the activation of cellular transcription factors such as

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NF-κB, NFAT, and AP-1 [8,9]. The mTOR inhibitor, rapamycin, has been shown to inhibit the progression of Kaposi's sarcoma [10]. Another human γ-Herpesvirus, Epstein-Barr virus (EBV), also possesses a constitutively active vGPCR, BILF1 [11]. EBV BILF1 can downregulate MHC-I/peptide complexes [12] and block the phosphorylation of RNA-dependent protein kinase (PKR) [13], thereby suppressing host antiviral responses. Therefore, γ-Herpesviral vGPCRs may confer diverse advantage in viral replication and dissemination during the life-long infection in a host. However, the molecular and structural details of how vGPCRs initiate and activate G-protein-coupled signaling remain largely unknown [1].

Herpesvirus *saimiri* (HVS) is a T cell lymphotropic virus and the prototype of the oncogenic γ2-herpesviruses (or rhadinoviruses), which also includes the human viral homologue, KSHV [15]. HVS, which were classified into three subgroups A, B, and C [16], is apathogenic in its natural host, the squirrel monkey, whereas experimental infection of HVS, especially subgroup C strains, in various primate species and rabbits causes acute and fatal T cell lymphoma [15]. In addition, HVS subgroup C strains can transform human T cells to continuously proliferate *in vitro* in the absence of antigenic or mitogenic stimulation [17]. Therefore, HVS has been considered as a good model system to study the mechanisms for transforming and pathogenic capability as well as apathogenic persistence of γ-herpesviruses in the host [15]. Similar to other γ-herpesviruses, HVS harbors various host cell modulatory genes, including a vGPCR [18]. Previously, it was reported that HVS vGPCR activates a broad range of signaling pathways through multiple G proteins [19]. However, the functional role of HVS vGPCR in viral pathogenesis and/or natural latency has been poorly defined, especially in T cells.

In this study, we investigated functional phenotypes of T cells expressing HVS vGPCR and found that the vGPCR can constitutively and potently activate T cells. Surprisingly, constitutive activation of T cells by the HVS vGPCR is independent of proximal T cell receptor (TCR) signaling molecules, such as TCRβ chain, Lck, and ZAP70, but requires a physical interaction with cellular CXCR4. We also show that HVS vGPCR constitutively down-regulates the chemokine receptor from the surface and sequesters it in the intracellular compartments. Although it was shown that vGPCR of EBV, a γ1-herpesvirus, physically interacts with CXCR4 and consequently inhibits CXCR4 signaling potentially via scavenging $G\alpha_i$ proteins [20], our unexpected findings on the positive cooperativity of HVS vGPCR with cellular CXCR4 in T cell activation shed light on the function of hetero-dimerization of vGPCR. In addition, the functional phenotypes induced by HVS vGPCR were reproducibly observed by KSHV vGPCR, but not by EBV vGPCR, suggesting that the functional activity of vGPCR is evolutionary conserved solely in γ2-herpesviruses. These results may not only provide new insight into the molecular basis of vGPCR in viral pathogenesis of γ2-herpesviruses but also open new avenues for studying the intermolecular interactions between GPCRs that lead to the activation of cellular signaling.

2. Materials and methods

2.1. Ethnics statement

Ethnical approval for using human peripheral blood monocytes from healthy volunteers was granted by the Institutional Review Board of Seoul National University Hospital (IRB No. C-1412-089-634) and all the experiments were carried out in accordance with the approved guidelines. All the healthy volunteers provided written informed consent prior to blood collection.

2.2. Cell culture and stimulation

Cultures of Human embryonic kidney (HEK) 293 T cells and HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. Jurkat T and SupT-1 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin, respectively. J.RT3-T3.5 (ATCC, TIB-153), J.CaM1.6 (ATCC, CRL-2063), P116 (ATCC, CRL-2676), and J.gamma1 (ATCC, CRL-2678) cells, which have deficient expression of TCRβ, Lck, ZAP70, and PLCγ1, respectively, were obtained from ATCC (www.atcc.org) and maintained in the same manner as Jurkat T cells. Specific deficiency of the indicated signaling proteins was confirmed by immunoblot using specific antibodies and whole cell lysates derived from the mutant cells (Supplementary Fig. S1). Lipofectamine2000 (Invitrogen) or calcium phosphate (Clontech) was used for transient expression of vGPCRs or CXCR4 in HeLa or 293T cells. Electroporation using a Bio-Rad electroporator (260 V, 975 μF) or Neon Transfection System (Invitrogen, 2150 V, 20 ms) was used for transient expression of vGPCR in Jurkat and SupT-1 cells. For TCR stimulation, Jurkat cells were incubated with anti-CD3/CD28 coated Dynabeads (ThermoFisher Scientific) for 18 h at 37 °C. In order to examine the effect of AMD3100 (Sigma-Aldrich), a CXCR4 antagonist, on the activation of T cells by vGPCR, Jurkat T cells were pre-incubated with 10 μM of AMD3100 for 30 min, and then cells were transduced with lentiviral vectors encoding vGPCR. Jurkat cells expressing vGPCR were analyzed for cellular activation after 24 h of incubation in the presence of AMD3100. Primary T lymphocytes were isolated from human peripheral blood mononuclear cells (PBMCs) by negative selection using the Miltenyi magnetically activated cell sorter (MACS) with pan T isolation kit (Miltenyi Biotec) and cultured in RPMI supplemented with 20% fetal bovine serum at a density of 1×10^6 cells/ml. Primary T lymphocytes were electroporated using Neon Transfection System (2150 V, 20 ms).

2.3. Plasmids and lentiviral vector

All the gene constructs for transient and stable expression in mammalian cells were cloned into pDEF3 GST [21], pHJEF IRES eGFP [22], pmCherry-C1 (Clontech), or pEF1α-IRES-Puro (Clontech). cDNAs encoding Human CXCR4 and EBV vGPCR were obtained from Addgene. HVS vGPCR was cloned from a HVS Bacmid clone (BAC43) which was kindly provided by Dr. Armin Ensser. Plasmid constructs encoding KSHV vGPCR were kindly provided by Dr. Pinghui Feng. The cDNA fragments containing full-length or mutant vGPCRs were sub-cloned into pHJEF IRES eGFP vector after PCR-based site-directed mutagenesis. The recombinant lentiviruses were obtained by co-transfection of lentiviral plasmids together with packaging vector psPAX2 and envelope vector pMD2.G into 293T cells. Cell culture supernatants containing the virus were collected at 48 h after transfection and used for lentiviral transduction.

2.4. Reporter assays

293T cells were transfected with plasmids encoding vGPCR together with the NFAT, AP-1, or NF-κB luciferase reporter plasmids (Stratagene). Plasmid encoding Renilla luciferase (pRL-CMV, Promega) was co-transfected as an internal control to evaluate transfection efficiency. At 48 h post-transfection, cells were washed once in ice-cold PBS and lysed in 200 μl of 1 × passive lysis buffer. Luciferase activity was assayed using the dual luciferase assay kit (Promega) according to the manufacturer's instructions. Luminescence was measured with 96 microplate luminometer (DTX880, Beckman Coulter Inc.) and normalized with Renilla luciferase activity.

2.5. Measurements of secreted IL-2

GFP-positive Jurkat cells transduced with lentiviral vector encoding vGPCR were sorted and incubated for 48 h. IL-2 secretion in the cell culture medium was measured with an enzyme-linked immunosorbent assay kit (PharMingen) according to the manufacturer's instruction.

2.6. Flow cytometry

Cells were washed with ice-cold fluorescence activated cell sorter (FACS) buffer (PBS containing 1% bovine serum albumin (BSA) and 1 mM EDTA), fixed with 4% paraformaldehyde, and subsequently stained with the following antibodies for 30 min at 4 °C: APC/Cy7-conjugated anti-CD69 (clone FN50, eBiosciences), PE/Cy7-conjugated anti-TCRαβ (clone IP26, eBiosciences), PerCP-Cy5.5-conjugated anti-CD4 (clone OKT4, eBiosciences), APC-conjugated anti-CXCR4 (clone 12G5, eBioscience), PE-conjugated anti-CD45 (clone HI30, BD Pharmingen), and PE-conjugated anti-CD25 (clone M-A251, BD pharmingen). For staining whole cellular CXCR4, cells were stained with APC-conjugated anti-CXCR4 antibodies, or isotype controls after fixation and perameabilization using Cytofix/Cytoperm solution (BD Biosciences). The cells were analyzed using a FACS Canto II flow cytometer (BD Biosciences). Data were analyzed by FlowJo software version 8.8.6 (FlowJo).

2.7. Immunofluorescence analysis and proximity ligation assay (PLA)

The cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature, and then permeabilized with 0.2% Triton X-100 for 15 min. After blocking with 5% BSA in PBS, the cells were incubated with mouse anti-Flag M2 (for vGPCR, Sigma) and rabbit anti-CXCR4 (clone 12G5, Abcam) antibody at 4 °C for 16 h in a humidifying chamber. Alexa488- or Alexa594-conjugated anti-mouse and anti-rabbit IgG antibodies (Molecular Probes) were used as secondary antibodies. A proximity ligation assay kit (Olink Bioscience Ltd.) was used to detect co-localization of vGPCR and endogenous CXCR4 *in situ* according to the manufacturer's instruction. Confocal microscopy was performed using an OlympusFV1000 laser-scanning microscope (Olympus). All images were analyzed and processed using the Olympus Fluoview or Adobe Photoshop software.

2.8. Pull-down assay, co-immunoprecipitation, and immunoblotting

Glutathione S-transferase (GST) pull-down assay was performed using lysate of Jurkat cells electroporated with pDEF3/vGPCR-GST construct [23]. In brief, 1×10^8 cells were harvested and lysed in NP-40 buffer (50 mM Tris–HCl [pH 7.4], 150 mM NaCl, 1% NP-40, 5 mM EDTA) supplemented with a protease inhibitor cocktail (Roche). Post-centrifuged supernatants were precleared with protein A/G beads (Santa cruz) at 4 °C for 2 h and then incubated with 50% slurry of glutathione-conjugated sepharose beads (Amersham Biosciences) at 4 °C for 4 h. Precipitates were washed extensively with lysis buffer. Proteins bound to glutathione beads were eluted with SDS-PAGE sample buffer after boiling at 70 °C for 5 min. For immunoprecipitation assay, 293T cells transfected with the indicated plasmids were harvested and lysed in 1 ml of RIPA buffer (50 mM Tris/HCl [pH 7.5], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS). The whole cell lysates were pre-cleared by centrifugation (15,000 ×*g*, 4 °C for 10 min) and then incubated with the indicated antibody and protein A/G-agarose beads at 4 °C overnight with gentle rotation. The immunocomplex was collected by centrifugation, washed 3 times with a buffer (0.1% Triton X-100, 50 mM Tris–Cl [pH 7.5], 150 mM NaCl, 1 mM EDTA), eluted with 2 × Laemmli sample buffer, and subjected to SDS-PAGE. Antibodies used in immunoblot analysis were as follows: mouse anti-FLAG M2 (Sigma), rabbit anti-CXCR4 polyclonal antibody (Abcam), goat anti-AU1-polyclonal antibody (Bethyl Laboratories), mouse anti-β-actin monoclonal antibody (Santa cruz), mouse anti-GAPDH monoclonal antibody (Calbiochem), rabbit anti-GST polyclonal antibody (Santa cruz), mouse anti-p38 monoclonal antibody (Santa cruz), mouse anti-phospo-p38 monoclonal antibody (Santa cruz), rabbit anti-PLCβ3 polyclonal antibody (Santa cruz), rabbit anti-phopho-PLCβ3 polyclonal antibody (Santa cruz), rabbit anti-Akt polyclonal antibody (Cell Signaling), rabbit anti-phospho-Akt polyclonal antibody (Cell Signaling), mouse anti-p44/42 MAPK (Erk1/2) monoclonal antibody (Cell Signaling), mouse anti-phospho-p44/42 MAPK (Erk1/2) monoclonal antibody (Cell Signaling), mouse anti-TCRβ (Thermo Scientific), mouse anti-Lck (Santa Cruz), rabbit anti-ZAP70 (Cell Signaling), and rabbit anti-PLCγ1 (Cell Signaling). Detection of protein bands was performed by using an enhanced chemiluminescence system (Pierce).

2.9. Measurement of intracellular calcium

Jurkat cells expressing vGPCR were loaded with Fluo-4AM (Molecular Probes) for 30 min at 37 °C. Cells were then washed and further incubated with fresh medium for 20 min. Live cells were analyzed with a FACS Canto II flow cytometer (BD Biosciences) and the fluorescence signal was quantified via FlowJo software version 8.8.6 (FlowJo).

2.10. CXCR4 knock-down in Jurkat cells

CXCR4-knockdown (K/D) in Jurkat cells was performed using a lentivirus system encoding shCXCR4 (clone ID., V2LHS_172391, Open Biosystems). The target sequence(ACAGCAACTAAGAACTTGG, located in 3′ untranslated region) of human CXCR4 was inserted into pGipz lentiviral vector. Lentiviruses were produced by transient transfection using packaging plasmids (psPAX2 and pMD2.VSV-G purchased from Addgene) after calcium phosphate-mediated transfection into 293T cells. Virus containing media were collected at 72 h post-transfection and used for transduction after passing the supernatants through a 0.45 μm filter. Nonsilencing-pGipz lentiviral shRNAmir control (RHS4346, Open Biosystems) construct was used as a negative control. Viral transduction was assessed by GFP expression and the transduced cells were selected by 2 μg/ml puromycin (Sigma-Aldrich). Stable knockdown of CXCR4 was confirmed by FACS analysis and immunoblotting for CXCR4.

2.11. Statistical analysis

Statistical analyses were performed using an unpaired two-tailed Student's *t*-test or one-way ANOVA with GraphPad Prism 6.0 software (GraphPad Software Inc.). Data are presented as the mean ± standard deviation. Statistical significance is defined at the $p < 0.05$ level.

3. Results

3.1. Constitutive activation of cellular signaling by γ2-herpesviral GPCRs

In order to confirm the constitutive activity of HVS vGPCR, we performed reporter assays driven by NFAT, AP-1, and NF-κB transcription factors in 293T cells (Fig. 1A). HVS vGPCR actively enhanced NFAT and AP-1-dependent gene expression in 293T cells in dose-dependent manner, but failed to induce the NF-κB-dependent reporter. It activated NFAT-mediated transcription as efficiently as KSHV vGPCR, which is highly and constitutively active [8,24], whereas its effect on AP-1-dependent transcription was relatively weaker than KSHV vGPCR. We further confirmed the activation of cellular signaling by HVS vGPCR in 293T cells and Jurkat cells by immunoblot (Fig. 1B). Transient overexpression of HVS vGPCR in both 293T and Jurkat cells induced phosphorylation of PLC-β, p38 MAP kinase, and AKT as efficiently as KSHV vGPCR did. Phosphorylation of ERK by HVS vGPCR expression was observed in Jurkat cells but not in 293T cells. Differential activation of ERK by KSHV vGPCR in a cell-type dependent manner has been previously reported [25]. Since activation of NFAT transcription factor is primarily mediated by the increased intracellular calcium, we also assessed cytosolic calcium with a fluorescent probe in Jurkat cells expressing HVS vGPCR. As expected, vGPCR expression increased cytosolic calcium level by 1.6 fold, compared to control cells (Fig. 1C and D). These results clearly show that HVS vGPCR, like KSHV vGPCR, is capable of constitutively activating cellular signaling.

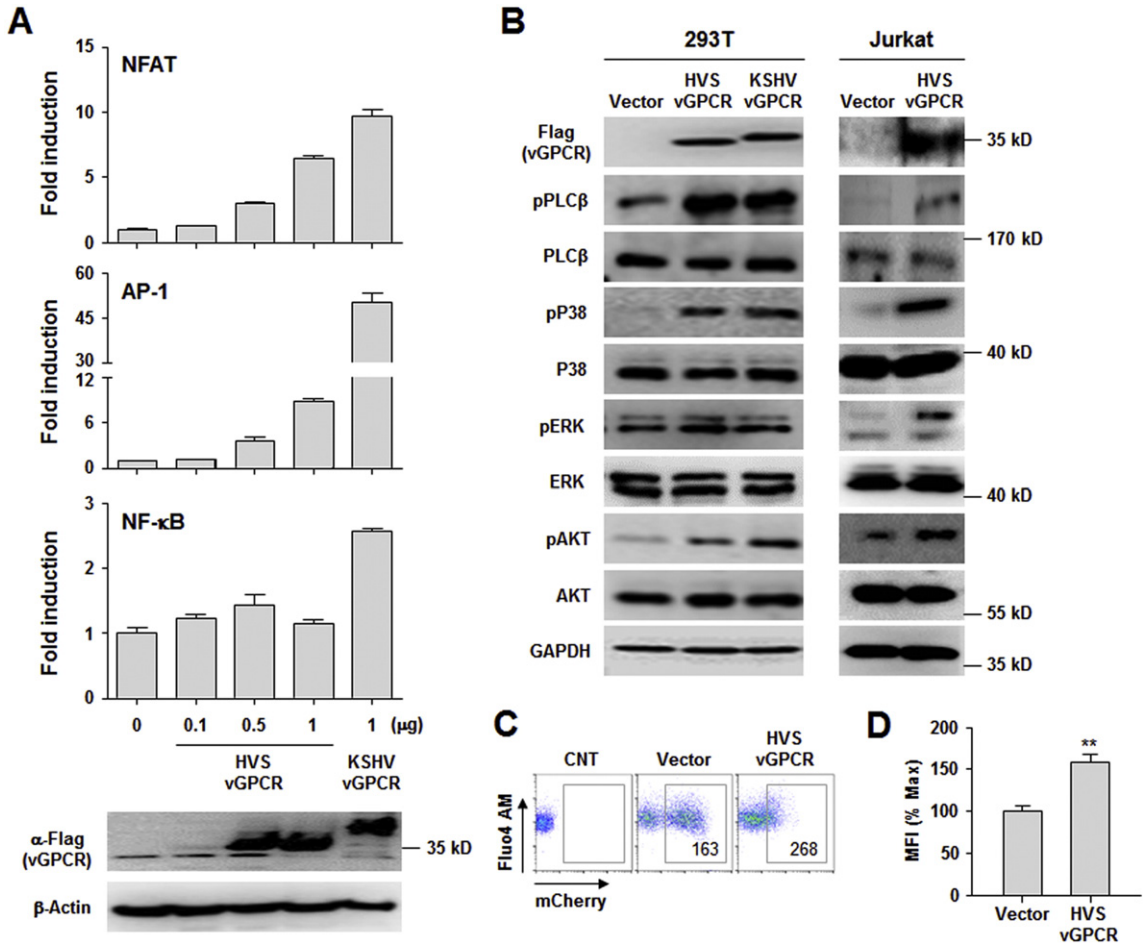

Fig. 1. HVS vGPCR constitutively activates cellular signaling. (A) 293T cells were transfected with the indicated reporter plasmid and increasing amounts of plasmid encoding HVS vGPCR. Activation of transcription factors was assessed by luciferase reporter assays. KSHV vGPCR was used as the positive control. Each assay was performed in triplicate and data are expressed as the mean + standard deviation. Representative immunoblots showing vGPCR expression and β-actin, as a loading control, are presented in the bottom panels. (B) Whole cell lysates of 293T cells or Jukat cells expressing vGPCR were analyzed by immunoblotting with antibodies against the indicated signaling proteins. Relative position of a molecular weight marker is indicated in each immunoblot. (C) and (D) Jurkat cells electroporated with HVS vGPCR fused with mCherry were loaded with Flor-4-AM and fluorescent intensities of mCherry-positive cells were quantified by flow cytomeric analysis at 48 h after electroporation. Mean fluorescent intensities of Flor-4-AM were presented within mCherry-positive gates (C). Each assay was performed in triplicate and data are expressed as the mean + standard deviation (D). **, $p < 0.01$.

3.2. Activation of T cells by γ2-herpesviral GPCRs

To investigate and compare the effect of γ-herpesviral vGPCRs on T cells, Jurkat cells were electroporated or lentivirally-transduced with plasmids encoding vGPCRs from HVS, KSHV, or EBV and CD69 surface expression was measured as an indicator of T cell activation. Expression of vGPCRs from HVS or KSHV, but not from EBV, significantly enhanced CD69 expression when compared to those of untransfected or vector-transfected cells (Fig. 2A and B, Supplementary Fig. S2). Activation of Jurkat cells by HVS or KSHV vGPCR was further confirmed by detection of IL-2 secretion (Fig. 2D) and elevation of IL-2 receptor α chain, CD25, on the surface (Supplementary Fig. S3). In addition to the T cell activation markers, we also examined other T cell receptors including TCR and CD4 and a chemokine receptor, CXCR4. Surface levels of TCR, CD4 as well as CXCR4 were substantially down-regulated in cells expressing vGPCR of HVS or KSHV, whereas expression of EBV vGPCR induced downregulation of CXCR4 but not TCR and CD4 (Supplementary Fig. S4 and Fig. 2A and B). Marginal changes in surface expression of CD45 were observed in cells expressing vGPCRs. In order to confirm the effect of vGPCRs on T cell activation, $CD3^+$ primary T cells purified from human PBMCs were electroporated with empty vector or plasmid encoding HVS vGPCR (Fig. 2D and E). Interestingly, activation of primary T cells, as measured by CD69 surface expression, was observed only in a fraction of cells where surface expression of CXCR4 was down-regulated (Fig. 2D). This negative correlation of CXCR4 down-regulation and CD69 up-regulation was also observed in Jurkat cells expressing vGPCRs of HVS or KSHV (Supplementary Fig. S2B). Cellular activation was reproducibly observed in another T cell line, SupT1 cells, by exogenous expression of HVS or KSHV vGPCRs, but not by EBV vGPCR (Supplementary Fig. S4C and S4D). Surface expression of CXCR4 was also down-regulated in SupT1 cells expressing vGPCRs. These results clearly indicate that expression of HVS or KSHV vGPCR can induce cellular activation of T cells, but EBV vGPCR failed to do so. In addition, changes in surface expression of CXCR4 or TCR/CD4 molecules in T cells suggest a potential link of those receptors with cellular activation by vGPCRs.

3.3. Upstream T cell receptor signaling complex is not required for cellular activation by HVS vGPCR

Since we observed concurrent downregulation of TCR and CD4 molecules in T cells by vGPCR expression, we next assessed the role of upstream TCR signaling components in T cell activation by using mutant Jurkat cell lines. Jurkat cell lines harboring mutations in TCRβ chain (J.RT3-T3.5), Lck (J.CaM1.6), ZAP70 (P116), or PLCγ1 (J.gamma1), were electroporated with empty vector or plasmid encoding HVS vGPCR and cellular activation was examined by measuring CD69 surface expression. As seen in Fig. 3, expression of HVS vGPCR in the mutant cell

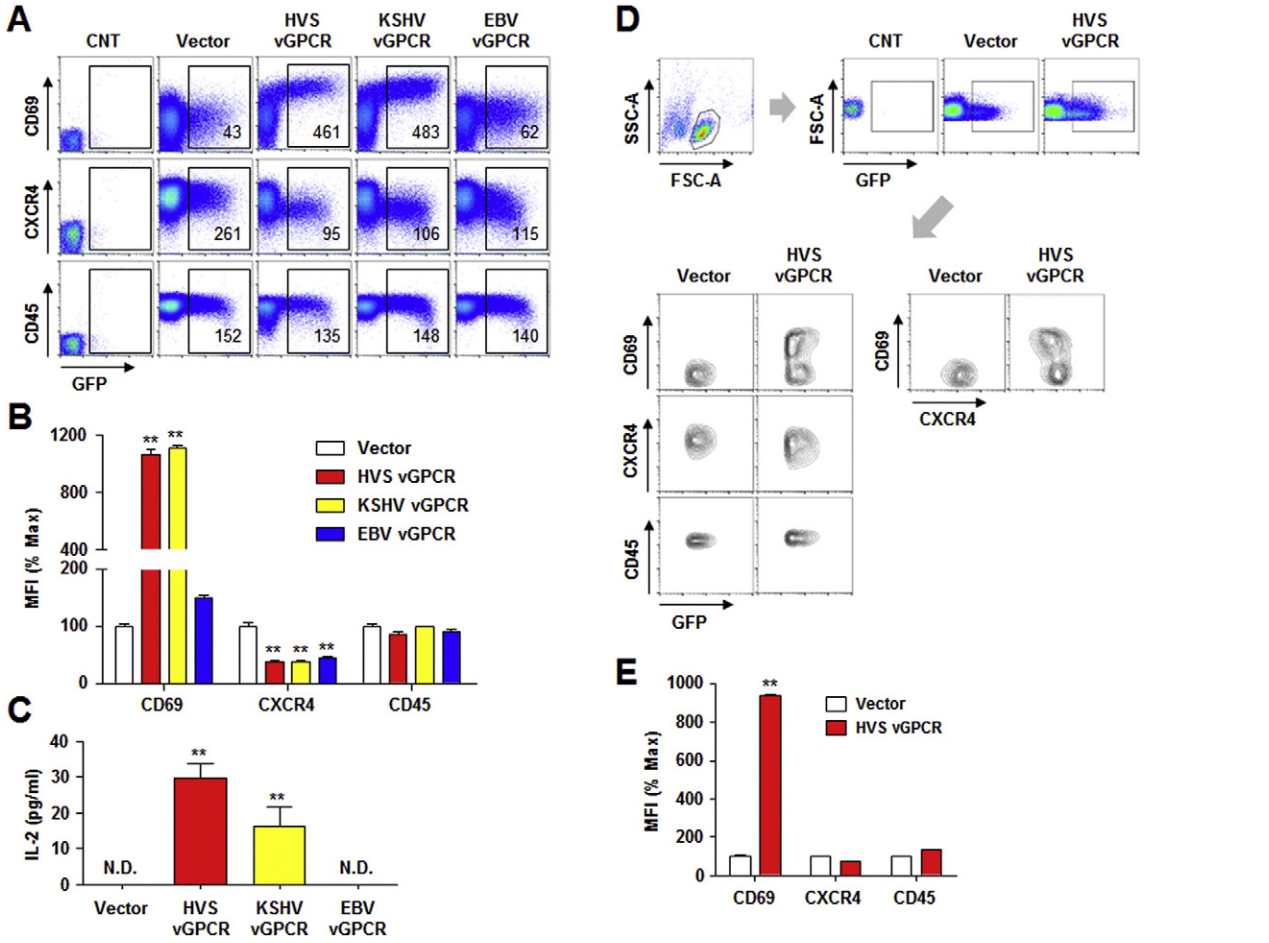

Fig. 2. HVS vGPCR constitutively activates T cells. (A) Jurkat cells were electroporated with pEF1α-IRES-eGFP plasmid encoding the indicated vGPCR and the surface level of cellular markers were analyzed in GFP-positive cells by flow cytometry at 48 h after electroporation. Mean fluorescent intensities of indicated markers were presented within GFP-positive gates. (B) Surface expression of the indicated cellular markers was quantitated from triplicate experiments in Jurkat cells expressing vGPCRs. Data are presented as the mean + standard deviation. (C) Production of IL-2 from Jurkat cells expressing vGPCRs was assessed by ELISA at 48 h after transduction with lentiviral vectors encoding the indicated vGPCR. This assay was performed in triplicate and data are presented as the mean + standard deviation. (D) Primary T cells isolated from human PBMCs were electroporated with pEF1α-IRES-eGFP plasmid encoding HVS vGPCR and the surface levels of cellular markers were analyzed in GFP-positive cells by flow cytometry at 48 h after electroporation. Gating strategy is presented in the upper panels. (E) Surface expression of the indicated cellular markers was quantitated from triplicate experiments in primary T cells expressing HVS vGPCR. Data are presented as the mean + standard deviation. **, $p < 0.01$ when compared to vector control.

lines lacking TCRβ, Lck, or ZAP70 could still induce cellular activation regardless of the mutations, indicating that the proximal components of early T cell receptor signaling are not required for T cell activation by HVS vGPCR. Interestingly, surface expression of CXCR4 was drastically down-regulated in the mutant cell lines upon vGPCR expression. In the absence of PLCγ1, however, the level of CD69 surface expression is lower than that of the other mutant cell lines, suggesting that PLCγ1 might be partially involved in the activation signaling mediated by vGPCR expression.

3.4. Involvement of CXCR4 in T cell activation by HVS vGPCR

Expression of vGPCRs in T cells not only induced T cell activation but also down-regulated surface expression of CXCR4. In addition, it was reported that herpesviral vGPCRs physically interact with cellular chemokine receptors including CXCR4 [1]. Therefore, we analyzed the potential role of cellular CXCR4 in T cell activation by HVS vGPCR. First, we examined whether HVS vGPCR physically interacts with CXCR4. As seen in Fig. 4A, vGPCR specifically interacted with cellular CXCR4 in GST-pull down assays. In addition, overexpressed HVS vGPCR primarily co-localized with cellular CXCR4 in both HeLa and Jurkat cells in intracellular compartments (Fig. 4B), indicating that they interact *in vivo*. The specific interaction of vGPCR and endogenous CXCR4 was further confirmed by an *in situ* proximity ligation assay (Fig. 4C) [26]. Based on the microscopic imaging assay, most CXCR4 complexed with vGPCR is localized in the cytoplasm, suggesting that interaction with vGPCR may induce endocytosis of CXCR4 or inhibit its trafficking to the cellular surface. When we examined the total protein level of CXCR4 in vGPCR-expressing Jurkat cells, there was no significant change in the amount CXCR4 with vGPCR expression (Supplementary Fig. S5). Therefore, expression of vGPCR in T cells may affect intracellular trafficking of CXCR4 via a physical interaction and thereby reduce its surface expression without significantly changing the protein level of the chemokine receptor. To investigate the functional role of CXCR4 in vGPCR-mediated T cell activation, we established a CXCR4-knockdown (K/D) Jurkat T cell line by using lentiviral shRNA targeting cellular CXCR4 and characterized it. Knockdown of surface expression as well as protein expression of CXCR4 was confirmed in the CXCR4 K/D Jurkat cells (Fig. 5A). CXCR4 K/D in Jurkat cells slightly enhances surface expression of TCR/CD3 molecules but does not significantly affect activation status as measured by CD69 expression (Supplementary Fig. S6) nor did it affect viability (data not shown). Although slightly decreased when compared to that of control cells, CXCR4 K/D Jurkat cells were also efficiently activated by stimulation with anti-CD3/CD28 coated beads, suggesting that their TCR signaling pathway remains intact (Supplementary Fig. S7). However, overexpression of HVS vGPCR tagged with mCherry severely impaired cellular activation in CXCR4 K/D cells compared to control cells (Fig. 5B and C). Similar results

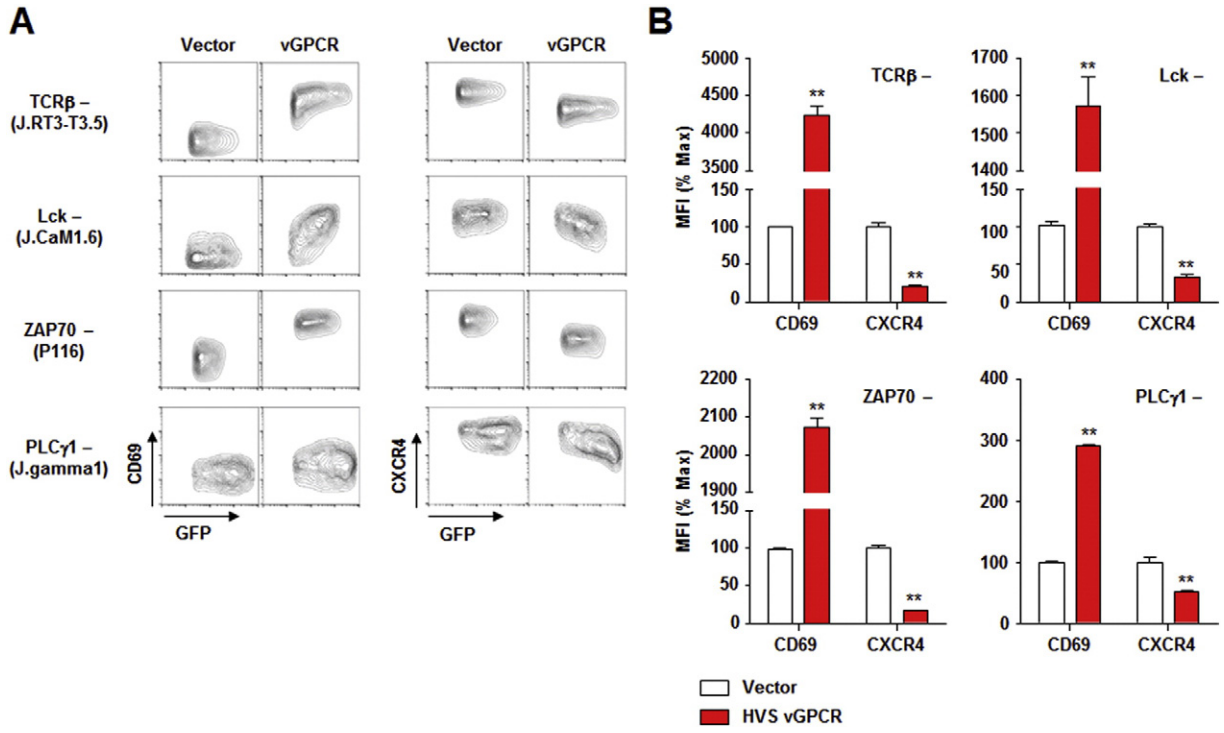

Fig. 3. Activation of T cells by HVS vGPCR is independent of proximal T cell signaling components. (A) Mutant Jurkat cell lines deficient in the indicated signaling molecules were electroporated with pEF1α-IRES-eGFP plasmid encoding HVS vGPCR and the surface levels of cellular markers were analyzed in GFP-positive cells by flow cytometry at 48 h after electroporation. (B) Surface expression of the indicated cellular markers was quantitated from triplicate experiments in the cell lines expressing HVS vGPCR. Data are presented as the mean + standard deviation. **, $p < 0.01$ when compared to vector control.

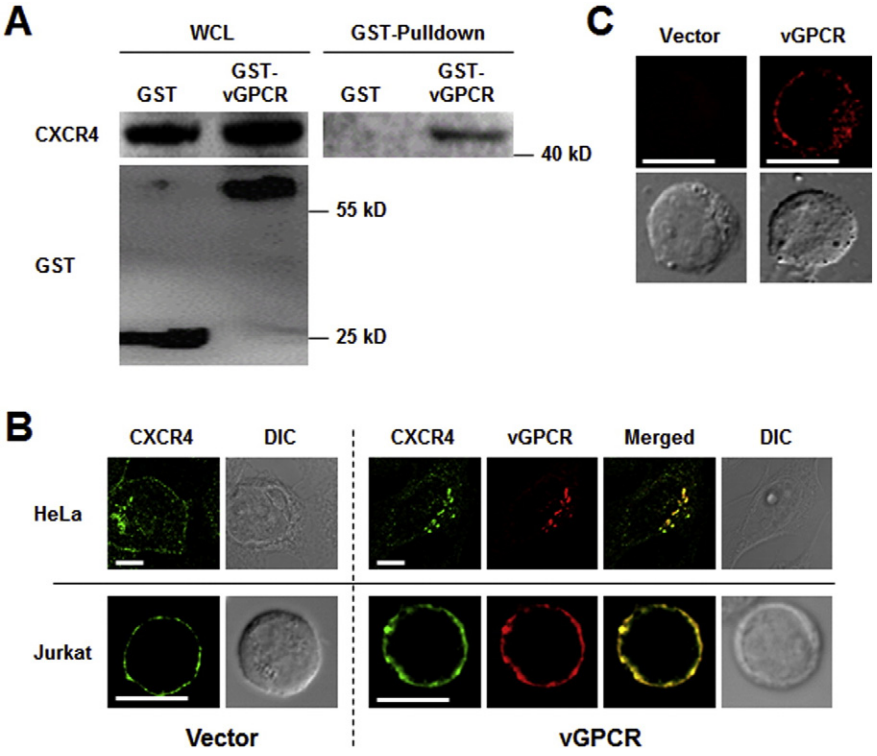

Fig. 4. HVS vGPCR interacts with cellular CXCR4. (A) Jurkat cells were electroporated with a plasmid encoding GST or GST-vGPCR and cellular lysates were applied for GST-pulldown assay. Interaction of vGPCR with cellular CXCR4 was assessed by immunoblot using an anti-CXCR4 antibody. Relative position of a molecular weight marker is indicated in each immunoblot. (B) HeLa and Jurkat cells expressing flag-tagged vGPCR were co-stained with antibodies against flag-tag (red) and cellular CXCR4 (green) and observed under confocal microscopy (right panels). Endogenous expression of CXCR4 in cells harboring empty vector was shown in left panels as control. White bar, 10 μm. (C) Jurkat cells electroporated with empty vector or plasmids encoding HVS vGPCR were analyzed after proximity ligation assay. Cells were stained with the specific antibody pairs against CXCR4 and flag and visualized by confocal microscopy. Representative image showing the signal (red) from the proximity ligation assay were presented. White bar, 10 μm.

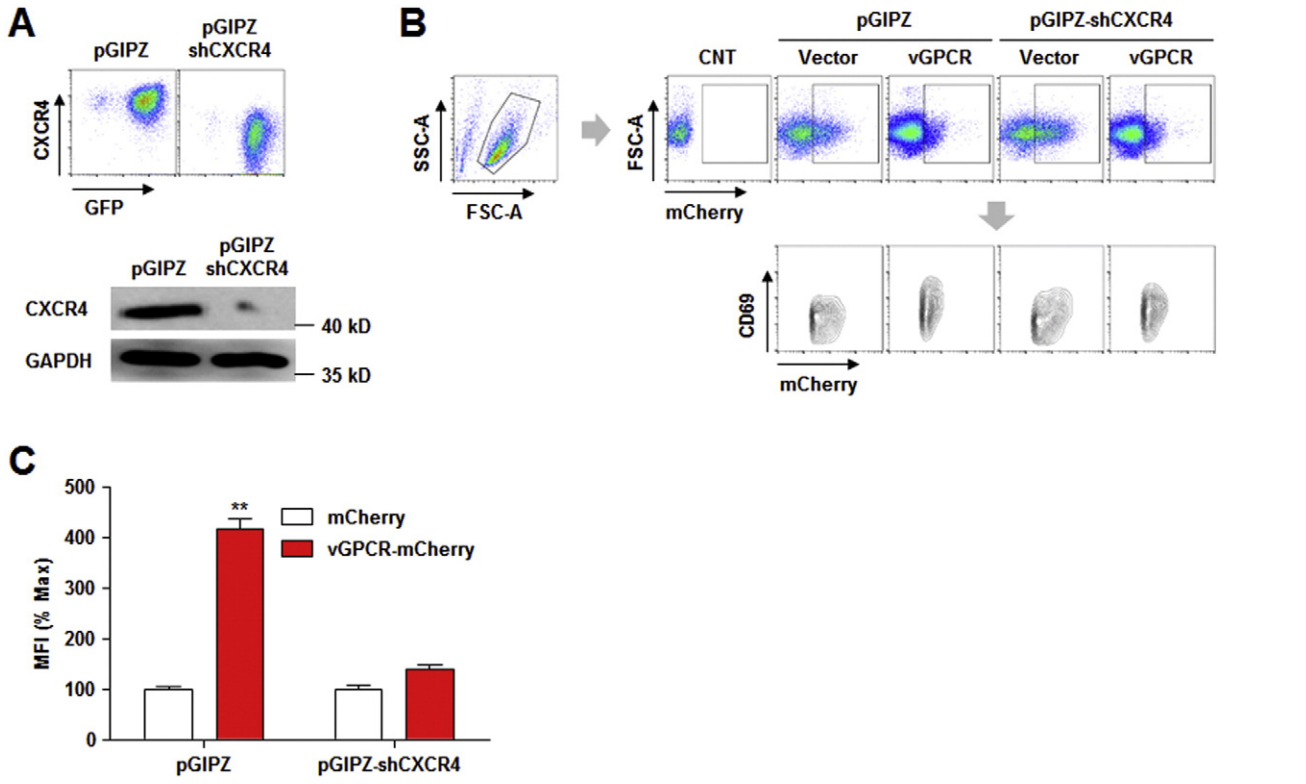

Fig. 5. CXCR4 silencing inhibits T cell activation by HVS vGPCR. (A) CXCR4 knockdown was performed using lentiviral vector encoding shRNA targeting CXCR4 and the surface expression (upper panels) and protein level (lower panels) of CXCR4 in the established Jurkat cell lines were analyzed by flow cytometry and immunoblot, respectively. Relative position of a molecular weight marker is indicated in each immunoblot. (B) CXCR4 knockdown Jurkat cells were electroporated with a plasmid encoding vGPCR fused with mCherry and the surface expression level of CD69 was assessed in mCherry-positive cells by flow cytometry at 48 h after the electroporation. Gating strategy is presented in the upper panels. (C) Surface expression of CD69 was quantitated from triplicate experiments in cells used in (B). Data are presented as the mean + standard deviation. **, $p < 0.01$ when compared to vector control.

were reproduced in CXCR4 K/D cells expressing KSHV vGPCR (Supplementary Fig. S8). In order to confirm whether complementation of CXCR4 could recover vGPCR-mediated T cell activation in CXCR4 K/D cells, cells were electroporated with plasmids encoding CXCR4 and HVS vGPCR tagged with mCherry. Expression of vGPCR-mCherry together with CXCR4 in CXCR4 K/D cells significantly enhanced CD69 expression (Supplementary Fig. S9). Taken together, these results indicate that expression of CXCR4 is required for T cell activation by HVS and KSHV vGPCRs.

3.5. Functional correlation of CXCR4 interaction with T cell activation by HVS vGPCR mutants

Constitutive activation of cellular signaling by vGPCR of KSHV is affected by several mutations at specific amino acid residues near or within the transmembrane domains as well as in its cytoplasmic C-terminus [27–29]. In order to further confirm whether constitutive activation of T cells by HVS vGPCR in the absence of ligand correlates with its interaction with CXCR4, we generated several specific point mutations at amino acid residues conserved in both HVS and KSHV vGPCRs, which have been shown to abolish constitutive activity of KSHV vGPCR without influencing vGPCR expression (Supplementary Fig. S10) [27–29]. The levels of expression of the mutant vGPCRs in Jurkat cells were not significantly different from those of wild type vGPCR (Fig. 6). Two mutations in transmembrane II of HVS vGPCR, L83D and L86D, which are equivalent to L91D and L94D mutations in KSHV vGPCR, respectively [27], failed to activate T cells and downregulate CXCR4 from the surface (Fig. 6A). In addition, the two mutant proteins did not interact with CXCR4 (Fig. 6B). L91D and L94D substitutions in KSHV vGPCR were shown to abolish its constitutive activity, while preserving its ability to be stimulated by an agonist, GROα [27]. One more mutant, R134A of HVS vGPCR (which is the equivalent of R143A in KSHV vGPCR, see Supplementary Fig. S9) [28], showed a phenotype similar to the L83D and L86D mutants when expressed in T cells (Fig. 6). The R143A substitution in KSHV vGPCR was shown to abolish its constitutive activity and responsiveness upon stimulation with GROα [28]. Another mutant, D75A of HVS vGPCR, the equivalent of D83A in KSHV vGPCR (Supplementary Fig. S9) [28], showed a similar level of T cell activation and CXCR4 downregulation as in wild type, and retained its ability to interact with CXCR4 (Fig. 6). It was shown that the D83A substitution in KSHV vGPCR could stimulate signaling activity 190% above wild type vGPCR, but poorly was stimulated by GROα [28]. A string of amino acids in the C-terminus of vGPCR is highly conserved in γ2-herpesviruses and is also involved in constitutive activation of G-protein mediated cellular signaling [29]. Therefore, we also generated mutations in the conserved C-terminus and investigate their effects on T cell activation as well as CXCR4 interaction. Deletion of the cytoplasmic C-terminus (Δ309–321) in HVS vGPCR abolished constitutive activation of T cells (Fig. 7A–C). The conserved QRM residues might be critical for T cell activation by vGPCR since partial deletion of the QRM sequence from the C-terminus (Δ309–314) failed to activate T cells, while partial deletion of the sequence beyond the conserved motif (Δ315–321) retained the ability for constitutive activation. Therefore, we next generated QRM/AAA or M311G mutations to further define the specific role of the amino acid residues in T cell activation (Fig. 7D and F). As reported in a previous study using KSHV vGPCR [29], mutations in cytoplasmic QRM or M311 residues in HVS vGPCR abrogated or dramatically reduced the constitutive activity of vGPCR. Moreover, the substitutions also abolished or diminished its interaction with CXCR4 (Fig. 7F). Taken together, these results showing that all point

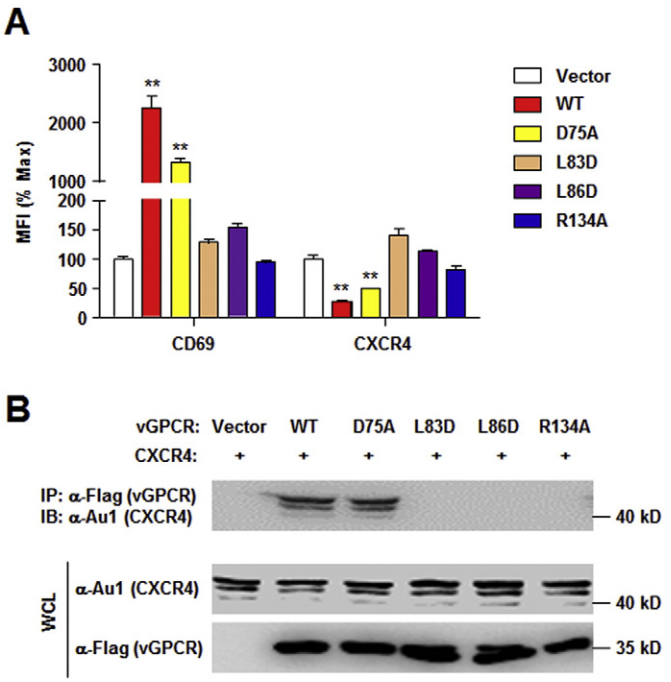

Fig. 6. Inactive mutant vGPCRs fail to interact with CXCR4. (A) Jurkat cells were electroporated with plasmids encoding wild type vGPCR or inactive mutants and examined for the surface expressions of cellular markers in triplicate experiments. Data are presented as the mean + standard deviation. **, $p < 0.01$ when compared to vector control. (B) 293T cells were transfected with plasmid encoding wild type vGPCR or the indicated mutants, together with a plasmid encoding CXCR4. Cellular lysates were precipitated with anti-flag antibody (vGPCRs) and applied for immunoblot with the indicated antibodies. Relative position of a molecular weight marker is indicated in each immunoblot.

mutations that failed to activate T cells were also unable to interact with CXCR4 strongly suggest that HVS vGPCR interaction with cellular CXCR4 is functionally linked to its constitutive activation of T cells.

4. Discussion

Since the discovery of the presence of three viral genes encoding homologs of cellular GPCRs in the genome of human cytomegalovirus (HCMV) in 1990 [30], vGPCRs have been identified in a number of β- and γ-herpesviruses as well as poxviruses [31]. vGPCRs are most homologous to chemokine receptors among host GPCR family members and appear to be exploited by the viruses for various pathogenic functions such as viral spread, immune evasion, and oncogenesis [31,32]. *Orf74* of Herpesvirus *saimiri* is a homolog of host CXCR2 [33] and the first viral vGPCR identified in oncogenic γ-herpesviruses [18]. Even though EBV and KSHV vGPCRs have been extensively characterized for their pathogenic roles in viral immune evasion and oncogenesis [2,32], the role of HVS vGPCR in viral pathogenesis remains poorly defined [19,33]. In addition, the effect of HVS vGPCR on cellular signaling in T cells has never been assessed even though HVS is a lymphotropic rhadinovirus (γ2-herpesvirus), like KSHV, and can cause fulminant T-cell lymphoma in non-natural host primates and transform human T cells *in vitro* [15]. Therefore, in the current study, we investigated the potential mechanisms exploited by HVS vGPCR in host T cells.

In a previous study, HVS vGPCR was shown to constitutively activate cellular signaling through $G\alpha_i$ and/or $G\alpha_{12/13}$, but not through $G\alpha_q$ [19]. The preferential activation of G protein subtypes by HVS vGPCR was validated by use of specific inhibitors such as pertussis toxin and C3 exoenzyme, which inhibit $G\alpha_i$- and $G\alpha_{12/13}$-dependent signaling, respectively. It was also reported that the vGPCR constitutively increased transcriptional activation of serum response element in 293T cells, but failed to induce the activity of cAMP response element-binding protein, NFAT, and NF-κB, which were only up-regulated in a ligand-dependent manner [19]. However, constitutive activation of NFAT-dependent transcription by HVS vGPCR was reproducibly observed in our experiments. In the previous study, Rosenkilde et al. used up to 50 ng (per 35,000 cells/96 well) of plasmid encoding vGPCR to show inducible activation of transcription factors by several vGPCR agonists [19], whereas we used up to 1000 ng (per 200,000 cells/24 well) of DNAs since we could not observe significant activation of the reporter system when the lower concentration of DNAs was used. Despite the discrepancy in the activation of NFAT transcription factor by HVS vGPCR in 293T cells, its potent activation of T cells, as measured by CD69 and CD25 upregulation as well as by IL-2 secretion, strongly suggests that it can stimulate cellular signaling mediated by NFAT in cooperation with AP-1, the primary transcriptional regulators of T cell activation [34]. Upstream signaling for NFAT activation includes the stimulation of phospholipase C (PLC), an enzyme that catalyzes the formation of inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) from phosphatidylinositol-4,5-bisphosphate (PIP2). IP_3 in turn activates the IP_3 receptor (IP_3R) on the endoplasmic reticulum (ER) to increase intracellular Ca^{2+} levels, resulting in activation of calcineurin phosphatase that dephosphorylates multiple phosphoserines on NFAT, leading to its nuclear translocation and activation [34]. GPCRs trigger a Ca^{2+} signal by activating PLCβ via the active $G\alpha_q$ subunit or Gβγ complex released by receptors that activate $G\alpha_i$ [35]. Increased cytosolic Ca^{2+} levels are then stabilized by activation of Ca^{2+} pumps in the ER (sarco/endoplasmic reticulum Ca^{2+} ATPase, SERCA) and the plasma membrane (plasma membrane Ca^{2+} ATPase, PMCA), which remove Ca^{2+} from the cytosol [35]. Previously, it was reported that activation of NFAT-dependent transcription by KSHV vGPCR is partially inhibited by the PLC inhibitor, U-73122, in 293T cells [36]. In addition, $G\alpha_i$ and $G\alpha_q$-coupled ERK1/2 pathways partially contribute to NFAT activation, as measured by specific inhibitors, in primary effusion lymphoma (PEL) cells expressing KSHV vGPCR [25]. However, Zhang et al. recently showed that KSHV vGPCR-induced NFAT activation is resistant to another PLC inhibitor, adelfosine, and an IP_3R inhibitor, 2-aminoethoxydiphenyl borate (2-APB), in 293T cells [9]. Instead, the vGPCR physically interacts with SERCA and inhibits its ATPase activity, thereby increasing cytosolic Ca^{2+} concentration and potentiating NFAT activation [9]. They also showed that cyclosporine A, a calcineurin inhibitor, treatment diminished NFAT-dependent gene expression and tumor formation induced by viral GPCRs *in vivo*, indicating that NFAT activation is essential in vGPCR-mediated tumorigenesis [9]. Consistent with this, we observed PLC-β activation as well as elevation of cytosolic Ca^{2+} levels in cells expressing HVS vGPCR (Fig. 1). Although we did not characterize the exact mechanism of how HVS vGPCR persistently activates the NFAT pathway, this might result from the direct activation of PLC-β or by inhibition of SERCA as with KSHV vGPCR [9].

The persistent activation of cellular signaling as well as transcriptional activation of NFAT and AP-1 by HVS vGPCR results in activation of T cells as measured by enhanced surface expression of activation markers, CD69 and CD25, as well as by IL-2 secretion. In a previous study that used a transgenic mouse model, CD2 promoter-driven expression of KSHV vGPCR in T cells induced angioproliferative lesions similar to human KS, potentially via an indirect and paracrine mechanism, although the phenotypes of cells expressing vGPCR were poorly defined [5]. In this study, we found that HVS vGPCR can activate Jurkat cells as well as primary human T cells. Interestingly, cellular activation is independent of cognate TCR signaling molecules, such as TCRβ, Lck, and ZAP70, but dependent on a chemokine receptor, CXCR4. The potential role of CXCR4 in T cell activation and modulation has been proposed by several studies [37–40]. Upon SDF-1, a cognate ligand of CXCR4, stimulation, CXCR4 physically interacts with TCR and utilizes the ZAP70-dependent pathway as well as the $G\alpha_{13}$-RhoA pathway for co-stimulatory signaling or cellular trafficking [39,41]. SDF-1 signaling via the CXCR4-TCR heterodimer uses PLCβ3 to activate the Ras-ERK pathway and increase intracellular calcium ion concentrations, whereas PLCγ1 is required for SDF-1-mediated migration via a mechanism

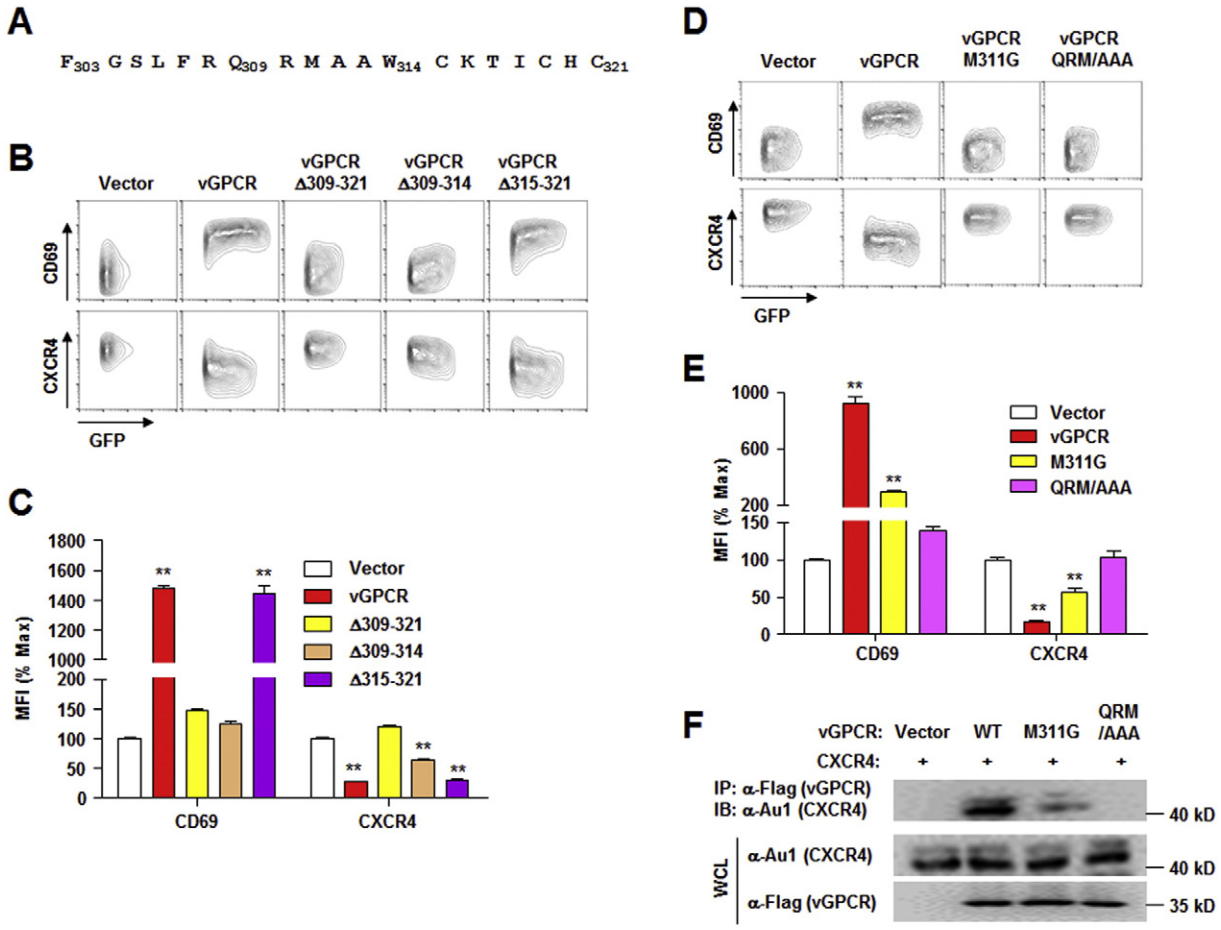

Fig. 7. Inactive mutations in cytoplasmic domain of vGPCR abrogate its interaction with CXCR4. (A) Amino acid sequence of the cytoplasmic tail of HVS vGPCR. (B) Jurkat cells were electroporated with plasmids encoding wild type vGPCR or the indicated mutants and examined for the surface expression of cellular markers in GFP-positive cells. (C) Surface expressions of the indicated cellular markers were quantitated from triplicate experiments in Jurkat cells used in (B). Data are presented as the mean + standard deviation. (D) Jurkat cells were electroporated with plasmids encoding wild type vGPCR or the indicated mutants and examined for the surface expression of cellular markers in GFP-positive cells. (E) Surface expressions of the indicated cellular markers were quantitated from triplicate experiments in Jurkat cells used in (D). Data are presented as the mean + standard deviation. (F) 293T cells were transfected with plasmid encoding wild type vGPCR or the indicated mutants, together with a plasmid encoding CXCR4. Cellular lysates were precipitated with anti-flag antibody (vGPCRs) and applied for immunoblot with indicated antibodies. Relative position of a molecular weight marker is indicated in each immunoblot. **, $p < 0.01$ when compared to vector control.

independent of LAT [40]. In addition, it was demonstrated that simultaneous expression and cooperation between CCR5 and CXCR4 are required for chemokine-induced T cell costimulation at the immunological synapse [37]. Therefore, it seems clear that CXCR4 contributes to T cell activation upon stimulation. In this study, however, we found that T cell activation by HVS vGPCR is mediated by a physical interaction, i.e. heteromeric complex formation, with cellular CXCR4 in the absence of SDF-1 stimulation and is independent of upstream TCR signaling molecules.

Then, how does the vGPCR and CXCR4 heteromeric complex stimulate cellular signaling to induce T cell activation? Crosstalk between GPCRs has been widely recognized to contribute to GPCR-mediated signaling events and functions [38,42–44]. The formation of homo- and/or hetero-oligomeric complexes among GPCRs opens a new dimension of possible molecular and functional GPCR interactions [38,42]. In addition to ligand-binding properties, unique allosteric interactions in GPCR homo- and/or hetero-oligomers may correlate with intrinsic or signaling efficacy [42]. For example, the functional interaction between EBV vGPCR, BILF1, with cellular GPCRs such as CXCR4 and histamine H4 receptor, has been reported and constitutively active vGPCR was shown to impair CXCR4 signaling upon SDF-1 stimulation by scavenging $G\alpha_i$ proteins [20]. One study also showed that KSHV vGPCR inhibits calcium mobilization induced by the thyrotropin-releasing hormone receptor and the muscarinic acetylcholine M1 receptor [45]. Although positive and negative regulation of cellular GPCRs, especially chemokine receptors, by other herpesviral vGPCRs have been reported in multiple studies [32], most studies mainly focused on the changes in signaling efficacy of cellular GPCRs upon ligand stimulation when co-expressed together with a vGPCR. In this study, we found that constitutive activation of T cells by HVS and KSHV vGPCRs is substantially abolished by knock-down of CXCR4, indicating that the interaction of the vGPCRs with cellular CXCR4 is functionally linked to the constitutive activity of vGPCR. In addition, functionally inactive vGPCR mutants are also unable to interact with CXCR4, suggesting that vGPCR function in T cells might be controlled through the formation of heteromeric vGPCR-CXCR4 complexes. Recently, it was reported that CXCR4 contributes to the function of the α_1-adrenergic receptor (AR), another GPCR, via heteromeric complex formation [38]. Disruption of the α_1-AR and CXCR4 heteromer by a peptide derived from the transmembrane helix 2 of CXCR4 or by CXCR4 knockdown abolished phenylephrine, an agonist of α_1-AR, induced calcium mobilization and myosin light chain phosphorylation, thereby inhibiting contraction of vascular smooth muscle cells upon α_1-AR activation [38]. Interestingly, a CXCR4 antagonist, AMD3100, did not affect phenylephrine-induced α_1-AR function, suggesting that heteromeric complex formation controls α_1-AR function independent of ligand occupation or the activation status of CXCR4 [38]. Dopamine receptor subtype-2 was shown to undergo similar phenomenon in a study that revealed that its function could be modulated by unoccupied ghrelin

receptor via formation of heteromeric complexes [46]. When we pretreated Jurkat cells with AMD3100, HVS vGPCR expression still activated T cells, but surface expression of CXCR4 was further downregulated (Supplementary Fig. S11). Therefore, the physical interaction with CXCR4 might be the primary requisite for the constitutive activity of HVS vGPCR, regardless of ligand occupation or the activation status of CXCR4. This hypothesis could be further supported by the results showing that all inactive vGPCR mutants examined failed to interact with CXCR4 (Fig. 6 and 7). Further analysis on the structural basis of the heteromeric complex needs to be conducted in order to explain how the specific amino acid residues mutated in the inactive forms contribute to heteromeric complex formation with CXCR4 as well as vGPCR activity. In addition, β-arrestin-mediated signaling, which can activate MAP kinases, such as ERK1/2 and p38, and AKT pathways [47,48], may play a role in continuous CXCR4 down-regulation from the cellular surface as well as concomitant signaling activation by vGPCR expression. However, the role of β-arrestin in vGPCR-mediated signaling remains to be determined [32].

Considering that CXCR4 is the chemokine receptor most widely expressed in malignant tumors and plays various roles in cellular proliferation, angiogenesis, and metastasis [48–50], exploitation of this chemokine receptor by vGPCR through heteromeric complex formation could be a novel strategy utilized by oncogenic γ2-herpesviruses such as KSHV and HVS. In our knowledge, this is the first report showing that a vGPCR potently activates T cells without involvement of proximal TCR signaling molecules, but in a cellular CXCR4-dependent manner. The physical contact potentially mediates allosteric interaction between the GPCRs [42,43] and might be a critical starting point initiating G-protein coupled signaling. Considering that EBV vGPCR fails to activate T cells, the functional impact on cellular activation by vGPCRs has been evolutionary conserved only in γ2-herpesviruses, but not in γ1-herpesviruses. Indeed, genomic location of the genes encoding vGPCRs in γ2-herpesviruses is conserved in *orf74*, whereas EBV gene, *bilf1*, encoding vGPCR is in a different position. KSHV and HVS vGPCR is homologous to cellular CXCR2 and might play more significant role in modulating cellular proliferation, but EBV vGPCR is more close to human CXCR4 and primarily involved in evading host immune responses by downregulating the surface MHC class I molecules and suppressing PKR [32]. Even though potential role of HVS vGPCR in viral replication or oncogenesis *in vivo* need to be confirmed by using a mutant virus, our current findings on the role of activation in host T cells provide valuable insight into the mechanisms controlling host cell signaling via intermolecular interactions between GPCRs.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2016.10.008.

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